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Research Article

Colletotrichum species (Glomerellales, Glomerellaceae) causing walnut anthracnose in China

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Abstract

Colletotrichum species can function as plant pathogens, saprobes or endophytes on a wide variety of plant hosts and are considered amongst the ten most significant genera of plant pathogens globally. China contributes almost half the walnut production in the world. However, *Colletotrichum* species occurring on walnut remain largely unresolved in China. To explore the *Colletotrichum* species found on walnut in China, 470 walnut fruit or leaf samples with anthracnose were collected from 14 main walnut-producing regions across seven provinces. A total of 165 *Colletotrichum* strains were isolated from these samples. The *Colletotrichum* isolates were identified, based on morphological characteristics and sequence analyses of *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2*. Twelve species, including 11 known *Colletotrichum* species (*C. boninense*, *C. citrulli*, *C. fioriniae*, *C. fructicola*, *C. godetiae*, *C. juglandicola*, *C. karsti*, *C. mengyinense*, *C. pandanicola*, *C. peakense* and *C. siamense*) and a novel species (*C. chinensis* **sp. nov.**) were identified. The species distribution revealed regional prevalence as follows: *C. mengyinense* was the most dominant species in Gansu, *C. mengyinense* and *C. siamense* in Shandong, *C. chinensis* in Beijing, *C. pandanicola* in Shaanxi and *C. godetiae* in Yunnan. *Colletotrichum siamense* was the sole species isolated in Sichuan and Xinjiang Provinces. Koch's postulates were fulfilled, demonstrating that all 12 species cause anthracnose on walnut. This is the first report of *C. boninense*, *C. citrulli* and *C. karsti* as pathogens of walnut anthracnose worldwide.

Key words: *Colletotrichum*, distribution, multi-gene phylogeny, pathogenicity, walnut anthracnose

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Introduction

Walnut (*Juglans regia* L., Juglandaceae) is an essential woody nut and oil crop cultivated worldwide, ranking first amongst the four nut types globally (Da Lio et al. 2018). Walnuts are popular fruits in China with a cultivation history of more than two thousand years (Guo 2016). In 2017, China contributed 47% of the global walnut production, maintaining the top global ranking since then (Liu et al. 2021). Due to their remarkable adaptability, extensive walnut-producing areas have been established across China (Pei and Lu 2011).

The genus *Colletotrichum* was introduced, based on the conidiomata with setae, with *C. lineola* Corda designated as the generic type (Corda 1831). The sexual morph of *Colletotrichum* was previously assigned to the genera *Gnomoniopsis* and *Glomerella* (Marin-Felix et al. 2017). With the implementation of "one fungus one name" nomenclature, *Colletotrichum* has been principally chosen to represent this genus (Réblová et al. 2016). More than 1,000 epithets have been accommodated within *Colletotrichum* (http://www.indexfungorum.org, accessed March 2024). Taxonomy of *Colletotrichum* species was confusing due to their morphological similarities and the absence of comprehensive studies using a polyphasic approach to differentiate between different species (Talhinhas and Baroncelli 2021). Morphological characteristics, however, had been considered useful in distinguishing species complexes (Cannon et al. 2012). For instance, the conidia of *C. acutatum* species complex tends to have acute ends or at least one acute end (Damm et al. 2012a), while the typical conidia of *C. boninense* species complex is cylindrical with a prominent basal scar (Damm et al. 2012b). Additionally, the conidia of *C. gloeosporioides* species complex tends to be cylindrical with round ends (Weir et al. 2012). A combination of morphological characteristics and multi-gene sequence analyses have been widely applied to define the taxonomic status in *Colletotrichum* species, which have been assigned into sixteen species complexes and some singleton species (Marin-Felix et al. 2017; Talhinhas and Baroncelli 2021; Liu et al. 2022).

Colletotrichum spp. comprised important plant pathogens, while some are endophytes or saprobes and could attack > 3,200 species of monocot and dicot plants (Cannon et al. 2012). Seventeen *Colletotrichum* species have been reported causing walnut anthracnose in the world, viz. *C. acutatum*, *C. aenigma*, *C. fioriniae*, *C. fructicola*, *C. gloeosporioides*, *C. godetiae*, *C. juglandicola*, *C. juglandis*, *C. kahawae*, *C. liaoningense*, *C. mengyinense*, *C. nymphaeae*, *C. pandanicola*, *C. peakense*, *C. siamense*, *C. sojae* and *C. viniferum* (He et al. 2019; Varjas et al. 2019; Mu et al. 2021; Wei et al. 2022; Cho et al. 2023; Li et al. 2023; Wang et al. 2023; Zhang et al. 2023). Several species of *Colletotrichum* have caused significant reductions in walnut production worldwide (Zhu et al. 2014; Wang et al. 2016; Da Lio et al. 2018). For instance, the causal agent of walnut anthracnose identified as belonging to the *Colletotrichum* genus led to 50–70% losses, with some walnut orchards experiencing 100% losses in nut production in France (Giraud and Verhaeghe 2015). *Colletotrichum nymphaeae* caused anthracnose on walnut in Brazil, destroyed approximately 50% of the fruits and the incidence was higher in rainy and hot summers (Savian et al. 2019).

In China, severe walnut anthracnose occurred in the orchards of Shandong Province, with the causal agents *C. gloeosporioides*, *C. siamense*, *C. fructicola* and *C. viniferum* (Zhu et al. 2014; Wang et al. 2017, 2018; He et al. 2019). The walnut leaf anthracnose caused by *C. fioriniae* led to severe losses in nut production in Hechi, Guangxi Province (Zhu et al. 2015). In addition, *Colletotrichum aenigma* caused severe fruit anthracnose in Hebei Province (Wang et al. 2021). *Colletotrichum nymphaeae* caused walnut branches anthracnose in Gansu Province (Ma et al. 2022). *Colletotrichum gloeosporioides*, *C. kahawae*, *C. nymphaeae*, *C. godetiae*, *C. fioriniae* and *C. juglandis* caused leaf spots of walnut in Hubei Province (Wei et al. 2022). Additionally, *Colletotrichum godetiae* caused severe anthracnose of walnut in Shaanxi and Yunnan Provinces with diseased fruits over 60% in the orchards (Wang et al. 2023).

Despite these studies on walnut anthracnose caused by *Colletotrichum* spp. from different regions in China, a comprehensive investigation into the species composition, geographic distribution and pathogenicity of these species is lacking. The aims of this study were to: (i) determine the species composition and geographic distribution of *Colletotrichum* spp. associated with walnut anthracnose in the principal production regions of China; and (ii) evaluate the pathogenicity of the *Colletotrichum* spp. by Koch's postulates.

Materials and methods

Sample collection and fungal isolation

During 2021–2023, a total of 470 fruit or leaf samples with anthracnose were collected from 14 primary walnut-producing areas in seven provinces (including Shandong, Yunnan, Sichuan, Shaanxi, Gansu, Xinjiang and Beijing) in China. Amongst these samples, there were 342 fruit samples and 128 leaf samples. Fragments (0.5 cm × 0.5 cm) of walnut, including the leaves and fruits, were cut aseptically from the margin of the disease lesion. The fragments were surface sterilised with 75% ethanol for 30 s, rinsed three times with sterile distilled water and dried on sterilised filter paper. Finally, the fragments were incubated on malt extract agar (MEA) for isolation of fungal strains (Fu et al. 2019). Petri dishes containing MEA with the fungal strains were incubated in the dark at 25 °C until the fungal colonies were observed. Hyphal tips resembling *Colletotrichum* colonies were transferred to Petri dishes with MEA.

DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelia grown on MEA plates with a CTAB plant genome DNA fast extraction kit (Aidlab Biotechnologies Co., Ltd, Beijing, China) and stored at -20 °C until further use. Five loci including the 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS), a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and partial actin (*ACT*), beta-tubulin (*TUB2*) and chitin synthase (*CHS-1*) were amplified using the primer pairs ITS1/ITS4 (White et al. 1990; Gardes and Bruns 1993), GDF1/GDR1 (Guerber et al. 2003), ACT-512F/ACT-783R (Carbone and Kohn 1999), T1/Bt2b (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997) and CHS-79F/CHS-345R (Carbone and Kohn 1999), respectively.

PCR amplification and sequencing were conducted following the protocols established by Fu et al. (2019). PCR amplicons were purified and sequenced at BGI Tech Solutions (Beijing Liuhe) Co., Limited (Beijing, China). Forward and reverse were assembled to obtain a consensus sequence using DNAMAN (v. 6.0.3.99; Lynnon Biosoft). Sequences generated in this study were deposited in GenBank (Suppl. material 1).

Phylogenetic analyses

DNA sequences of concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* loci were analysed to investigate the phylogenetic relationships amongst *Colletotrichum*

species with DNA sequences available from GenBank (http://www.ncbi.nlm.nih.gov/genbank/accessed March 2024) (Suppl. material 1). Multiple sequences were aligned using the MAFFT v.7.110 (http://mafft.cbrc.jp/alignment/server/ accessed March 2024) and adjusted manually in MEGA v.7.0 (Kumar et al. 2016). Gaps were manually adjusted to optimise the alignment (Tamura et al. 2013).

Phylogenetic analyses of Maximum Likelihood (ML), Bayesian Inference (BI) and Maximum parsimony (MP) were performed. Maximum Likelihood analyses were constructed on the RAxML-HPC BlackBox 8.2.10 (Stamatakis 2014) using the GTR+GAMMA model with 1,000 bootstrap replicates. The Bayesian phylogenetic analysis was performed using a Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.2.6 (Ronquist et al. 2012). Four MCMC chains were run from random trees and trees were sampled by each 1,000th generation. The first 25% of the trees of MCMC sampling were discarded as burn-in and posterior probabilities (PP) were determined from the remaining trees. Maximum parsimony (MP) analysis, based on the concatenated dataset, was conducted in PAUP* v. 4.0b10 with the default options (Swofford 2002). Ambiguous regions in the alignment were excluded and gaps were treated as missing data. Clade stability was evaluated in a bootstrap analysis with 1,000 replicates with max-trees set to 1,000 with other default parameters used as implemented in PAUP* (Hillis and Bull 1993). Other measures used to evaluate parsimony scores included the consistency index (CI), rescaled consistency (RC), homoplasy index (HI) and retention index (RI). The phylogenetic trees were configured in FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and edited using Adobe Illustrator CC2020 (Adobe Systems Inc., USA).

Morphological characterisation

To assess the colony characteristics, mycelial plugs (8 mm in diameter) were transferred from the growing edges of 7-day-old colonies on to PDA and MEA and incubated at 25 °C in darkness. Colony diameters were measured after 7 days of incubation and were used to calculate the mycelium growth rate (Zhang et al. 2023). Morphology and colony characteristics were determined following the methods described by Damm et al. (2012a). Appressoria were induced on slide cultures according to the protocol established by Weir et al. (2012). The shape, colour and size of conidia, conidiophores, setae, conidiogenous cells and appressoria were measured by at least 20 measurements using a microscope (Nikon Eclipse E600) (Zhang et al. 2023). Fungal isolates and specimens were deposited at Beijing Forestry University, with duplicates at the China General Microbiological Culture Collection Center (CGMCC; https://www.cgmcc.net/english, accessed March 2024).

Prevalence

To determine the abundance of *Colletotrichum* species in sampled provinces, the isolation rate (R^I) for each species was calculated using the formula: $R^I\ \% = (N^s/N^t) \times 100$, where N^s represents the number of isolates from the same species and N^t is the total number of isolates from each sample-collected province (Fu et al. 2019).

Pathogenicity test and virulence on walnut tissues

The pathogenicity of all isolated species was examined on walnut fruits and leaves. Mycelial plugs derived from representative isolates obtained in this study were utilised for pathogenicity test. Isolates of all species were incubated on MEA plates for 7 days prior to inoculation.

The pathogenicity test was performed on detached living walnut fruits and leaves. Briefly, fruits and leaves were washed with sterilised water and surfaces sterilised with 75% ethanol for 1 min. The fruits and leaves were inoculated using the spore suspension and non-wound inoculation methods (Fu et al. 2019; Zhang et al. 2023). For inoculation, an aliquot of 20 µl of spore suspension (1.0×10^6 conidia per ml) was inoculated on to fruits and leaves without wounding them. Eight replicates were used for each treatment. Fruits and leaves, inoculated by sterilised water, served as the negative control. The inoculated detached fruits and leaves were incubated under 25 °C with 12/12 h light/dark photoperiod. Pathogenicity was determined by measuring the lesion length of fruits and leaves after 10 days' incubation. To fulfil Koch's postulates, the fungal pathogens were re-isolated from the lesion and identified, based on morphology and DNA sequences.

Mean comparisons were conducted using Tukey's honest significant difference (HSD) test ($\alpha = 0.05$) in R (Version 3.2.2, R Inc. Auckland, NZL).

Results

Colletotrichum species associated with walnut anthracnose

During 2021–2023, a total of 342 fruit samples and 128 leaf samples from diseased walnut trees exhibiting anthracnose were collected in seven provinces (Beijing, Shandong, Yunnan, Sichuan, Shaanxi, Gansu and Xinjiang) of China. (Figs 1, 2). A total of 165 *Colletotrichum* strains were isolated from these samples. The occurrence of 12 *Colletotrichum* species is summarised in Suppl. material 2.

Multi-locus phylogenetic analyses

Based on the results of BLAST in GenBank, 31 representative isolates together with 149 previously described species (Suppl. material 2) were subjected to multi-locus phylogenetic analyses with concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* sequences for those belonging to the *C. gloeosporioides*, *C. boninense* and *C. acutatum* species complex. The results indicated that these 31 isolates clustered together with 12 species (Figs 3–5).

The concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* dataset (1,772 characters with 251 parsimony-informative characters) from 55 ingroup isolates of *Colletotrichum acutatum* species complex was used for phylogenetic analysis. The heuristic search with random addition of taxa (1,000 replicates) generated 5,000 most parsimonious trees (Length = 962, CI = 0.671, HI = 0.329, RI = 0.830, RC = 0.557). The topologies obtained from the Maximum Parsimony, Maximum Likelihood and Bayesian analysis were comparable. In three analyses (ML, Bayesian and MP), four isolates clustered in two clades corresponding to *C. fioriniae* (2 isolates) and *C. godetiae* (2 isolates) (Fig. 3).

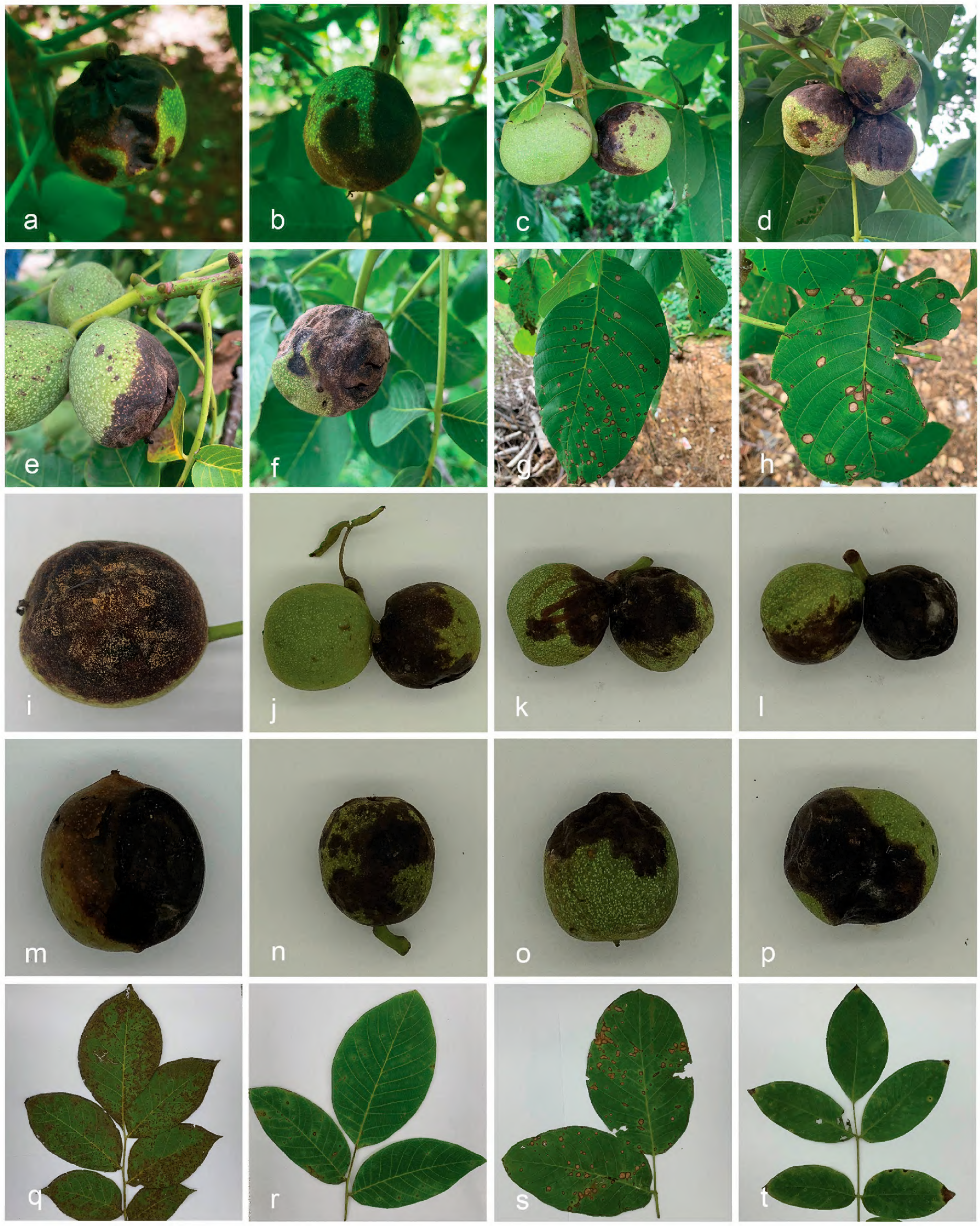

Figure 1. Representative symptoms of walnut anthracnose on leaves and fruits **a–f, i–p** symptoms on fruits of *Juglans regia* **g, h, q–t** symptoms on leaves of *Juglans regia*.

The concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* dataset (1,875 characters with 339 parsimony-informative characters) from 39 ingroup isolates of *Colletotrichum boninense* species complex was used for phylogenetic analysis.

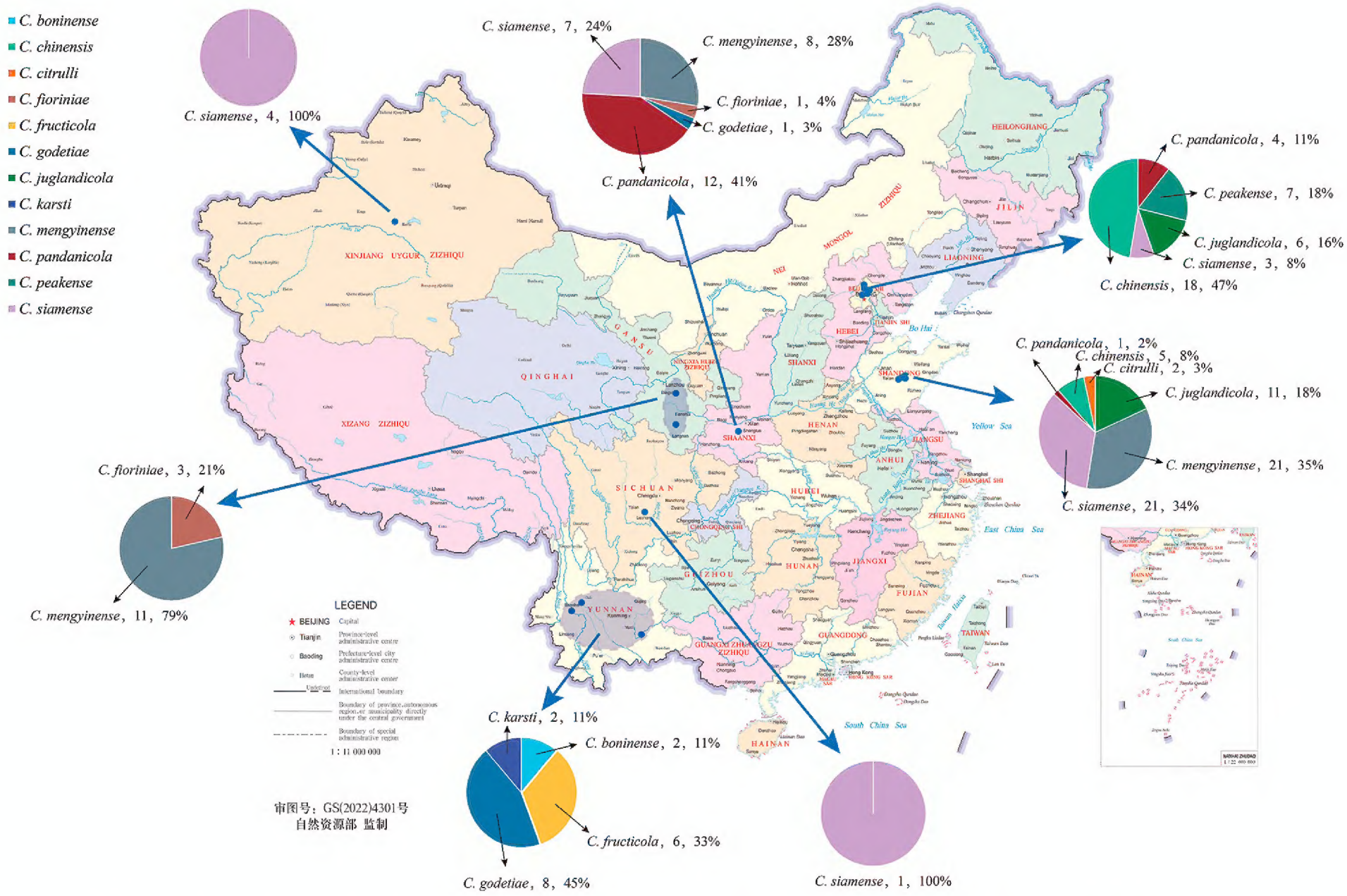

Figure 2. Map of China indicating locations where walnut trees were sampled and the species of *Colletotrichum* obtained from each province. The 12 species of *Colletotrichum* are indicated.

The heuristic search with random addition of taxa (1,000 replicates) generated 5,000 most parsimonious trees (Length = 1283, CI = 0.672, HI = 0.327, RI = 0.728, RC = 0.490). The topologies obtained from the Maximum Parsimony, Maximum Likelihood and Bayesian analyses were comparable. In three analyses (ML, Bayesian and MP), four isolates clustered in two clades corresponding to *C. boninense* (2 isolates) and *C. karsti* (2 isolates) (Fig. 4).

The concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* dataset (1,959 characters with 372 parsimony-informative characters) from 106 ingroup isolates of *Colletotrichum gloeosporioides* species complex was used for phylogenetic analysis. The heuristic search with random addition of taxa (1,000 replicates) generated 5,000 most parsimonious trees (Length = 1510, CI = 0.580, HI = 0.420, RI = 0.811, RC = 0.470). The topologies obtained from the Maximum Parsimony, Maximum Likelihood and Bayesian analysis were comparable. In the phylogenetic tree constructed for the isolates in the *C. gloeosporioides* complex, 23 isolates clustered in eight clades corresponding to *C. citrulli* (2 isolates), *C. fructicola* (2 isolates), *C. juglandicola* (2 isolates), *C. mengyinense* (4 isolates), *C. pandanicola* (4 isolates), *C. peakense* (2 isolates) and *C. siamense* (3 isolates). Noticeably, four isolates (CGMCC 3.25209, CGMCC 3.25210, CGMCC 3.25211 and CGMCC 3.25212) clustered distantly from any known species in the complex and are herein described as a new taxon, namely *C. chinensis*, based on the guidelines established in Chethana et al. (2021) and Jayawardena et al. (2021) (Fig. 5).

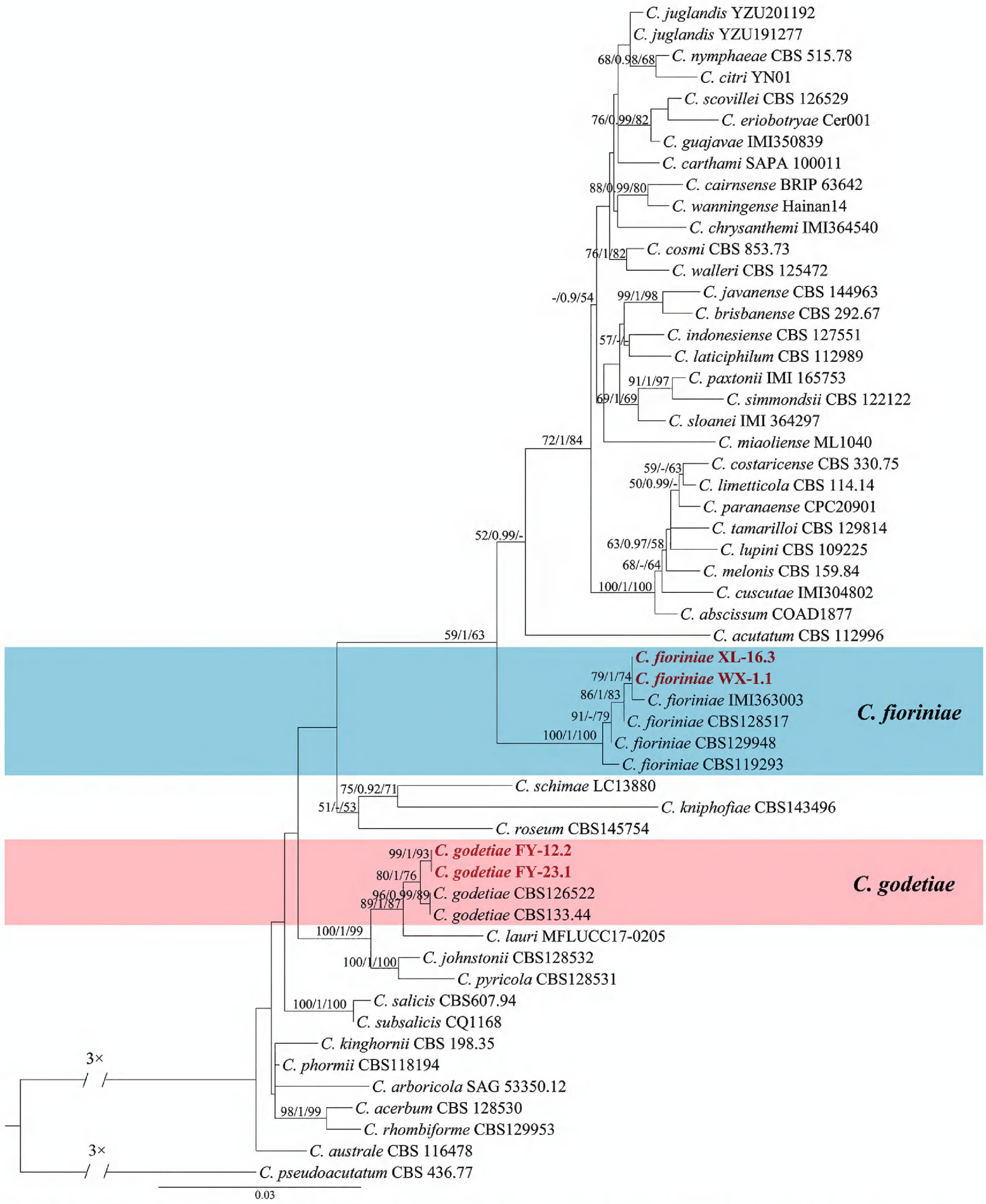

Figure 3. Maximum Likelihood tree generated from sequence analysis of the concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* genes dataset of *C. acutatum* species complex. The species *C. pseudoacutatum* CBS 436.77 was selected as an outgroup. Bayesian posterior probability (PP ≥ 0.90), MP bootstrap support values (ML ≥ 50%) and RAxML bootstrap support values (ML ≥ 50%) were shown at the nodes (ML/PP/MP).

Morphological investigation

Colonies of the representative isolates were selected to observe their morphological characteristics (Suppl. materials 3, 4). Some differences in colony morphology were obviously observed amongst the 12 species. Abundant

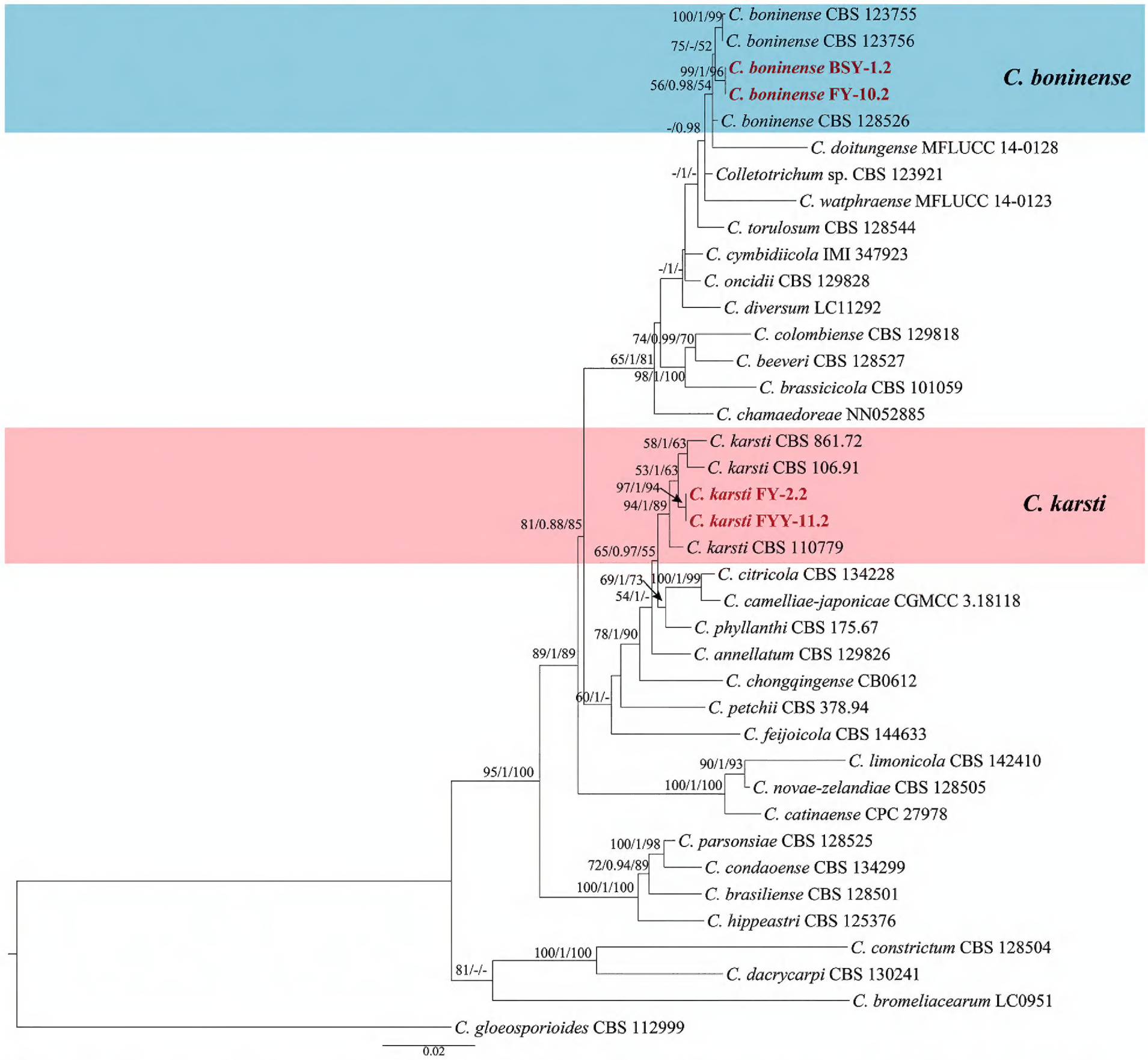

Figure 4. Maximum Likelihood tree generated from sequence analysis of the concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* genes dataset of *C. boninense* species complex. The species *C. gloeosporioides* CBS 112999 was selected as an outgroup. Bayesian posterior probability (PP ≥ 0.90), MP bootstrap support values (ML ≥ 50%) and RAxML bootstrap support values (ML ≥ 50%) were shown at the nodes (ML/PP/MP).

orange conidial masses were often observed in the centre of the colonies. A significant difference in growth rate across 12 *Colletotrichum* species was observed (Suppl. material 4). Colonies diam. on MEA, the mycelial growth rate of *C. mengyinense* was the highest, with an average of 80.3 ± 0.6 mm after 7 days. This was followed by *C. citrulli* (78.8 ± 6.3 mm), *C. pandanicola* (76.3 ± 1.5 mm), *C. peakense* (76.3 ± 2.6 mm) and *C. chinensis* (75.7 ± 0.6 mm). The growth rate of *C. godetiae* was the slowest (48.7 ± 2.1 mm) (Suppl. material 4). Colonies diam. on PDA, the mycelial growth rate of *C. mengyinense* was the highest, with an average of 82.3 ± 1.5 mm after 7 days, followed by *C. siamense* (82.0 ± 0 mm) and *C. chinensis* (80.0 ± 0 mm). The growth rate of *C. juglandicola* was the slowest (49.1 ± 1.9 mm) (Suppl. material 4).

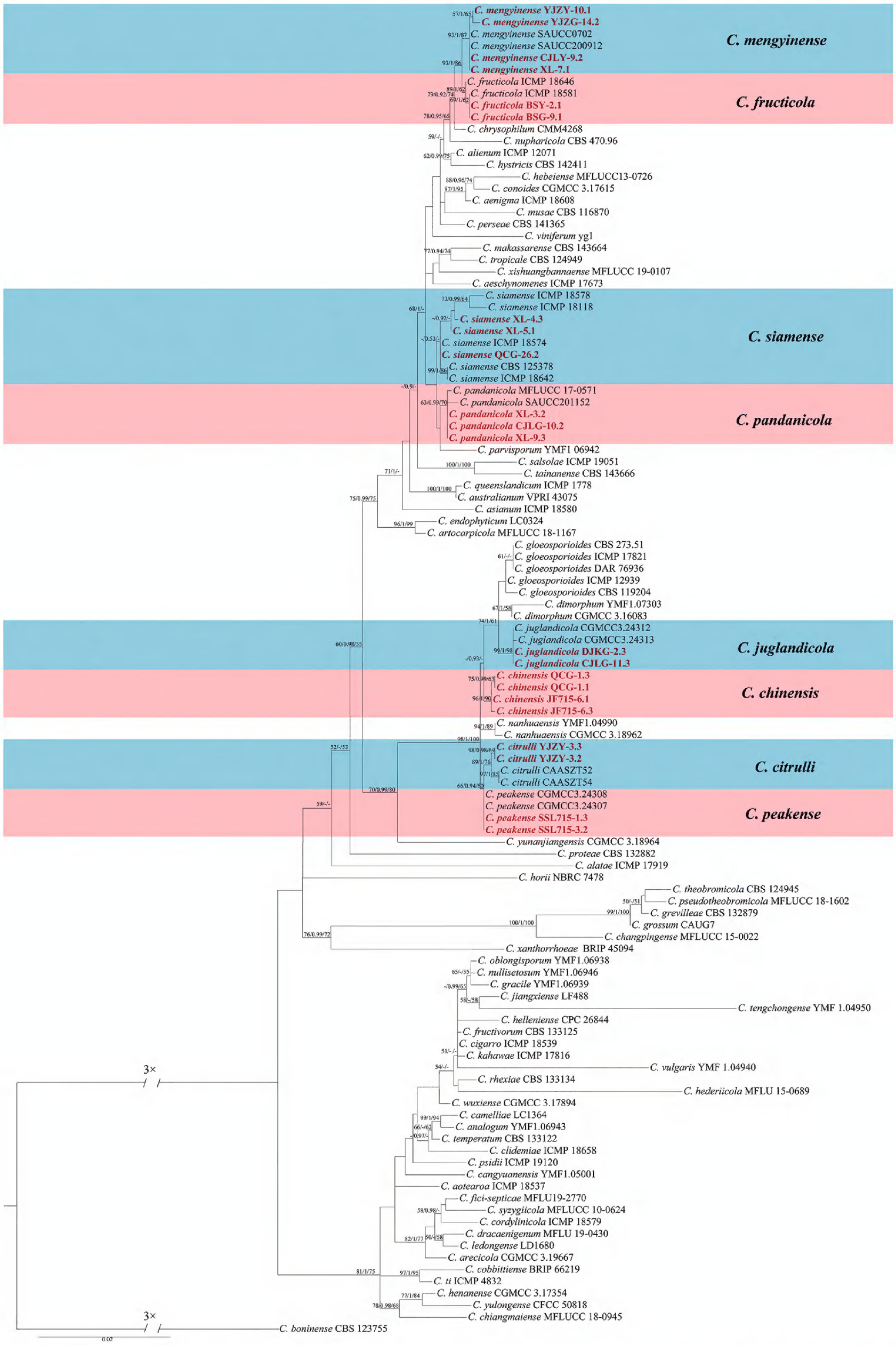

Figure 5. Maximum Likelihood tree generated from sequence analysis of the concatenated *ACT*, *CHS-1*, *GAPDH*, ITS and *TUB2* genes dataset of *C. gloeosporioides* species complex. The species *C. boninense* CBS 123755 was selected as an outgroup. Bayesian posterior probability (PP ≥ 0.90), MP bootstrap support values (ML ≥ 50%) and RAxML bootstrap support values (ML ≥ 50%) were shown at the nodes (ML/PP/MP).

Colletotrichum chinensis **Y. Zhang ter & L. Zhang, sp. nov.**
Index Fungorum: IF901166
Facesoffungi Number: FoF14886
Fig. 6

Holotype. QCG-1.

Etymology. Named after China where the fungus was collected.

Description. Associated with walnut fruit and leaf anthracnose. Sexual morph not observed. Asexual morph developed on MEA. ***Conidiomata*** acervular, conidiophores hyaline, smooth-walled, septate, branched. ***Setae*** medium to dark brown, smooth to finely verruculose close to the tip, the tip rounded, 1–3 aseptate, 39.2–118.7 µm long. ***Conidiogenous cells*** subcylindrical, straight to curved, 16.7–30.0 × 2.3–3.7 µm (mean ± SD = 22.2 ± 0.6 × 3.2 ± 0.1 µm, n = 30). ***Conidia*** hyaline, smooth-walled, subcylindrical, both ends round, 1–3-guttulate, contents granular, 13.7–18.5 × 4.4–5.9 µm (mean ± SD = 16.4 ± 1.0 × 5.0 ± 0.3 µm, L/W radio = 3.3, n = 100).

Culture characteristics. Colonies on MEA flat with entire margin, surface pale pink, covered with felty white aerial mycelium aerial; reverse rosy buff to honey-coloured, growth rate 75–76 mm diam. in 7 d. Colonies on PDA flat with entire margin, surface pale pink, covered with felty white or grey aerial mycelium, grey aerial mycelium in the centre; reverse buff, rosy buff to honey-coloured, growth rate 79–80 mm diam. in 7 d. ***Appressoria*** produced on slide culture from conidia, medium to dark brown, variable in shape, often smooth-walled, subglobose, ovate to broadly elliptical in outline, 7.3–12.0 × 4.7–6.7 µm (mean ± SD = 9.5 ± 0.2 × 5.8 ± 0.1 µm, L/W radio = 1.6, n = 40).

Material examined. CHINA, Shandong Province, Taian City, on fruit of *Juglans regia* L., 29 July 2022, Y. Zhang, L. Zhang and L.L. Zhao (holotype, QCG-1, culture extype, QCG-1.1 = CGMCC 3.25209; culture QCG-1.3 = CGMCC 3.25210); Beijing City, on fruit of *Juglans regia* L., 15 July 2022, Y. Zhang, L. Zhang and L.L. Zhao (JF715-6, culture, JF715-6.1 = CGMCC 3.25211; culture, JF715-6.3 = CGMCC 3.25212).

Notes. Phylogenetic analysis of the concatenated set of nucleotides from five loci indicated that *Colletotrichum chinensis* nested in the clade of *C. gloeosporioides* species complex and was closely related to *C. citrulli*, *C. dimorphum*, *C. gloeosporioides*, *C. juglandicola*, *C. nanhuaensis* and *C. peakense* (Cannon et al. 2008; Guo et al. 2022; Yu et al. 2022; Zhang et al. 2023). Morphologically, the strikingly longer conidia or appressoria of *C. chinensis* could be readily distinguishable from *C. citrulli*, *C. dimorphum*, *C. gloeosporioides*, *C. juglandicola*, *C. nanhuaensis* or *C. peakense*. *Colletotrichum citrulli*, *C. dimorphum* and *C. nanhuaensis* were originally reported from *Ageratina Adenophora* (Spreng.) King & H.Rob. and *Citrullus lanatus* (Thunb.) Matsum & Nakai, respectively in China (Guo et al. 2022). *Colletotrichum juglandicola* and *C. peakense* had been reported from *Juglans regia* L. as new species in China (Zhang et al. 2023). Thus, *Colletotrichum chinensis* was identified as a new species in this study, which caused anthracnose of *Juglans regia*.

Prevalence

In this study, *Colletotrichum mengyinense* was the dominant species (40/165, 24.2%), followed by *C. siamense* (36/165, 21.8%), *C. chinensis* (23/165, 13.9%), *C. pandanicola* (17/165, 10.3%), *C. juglandicola* (17/165, 10.3%), *C. godetiae*

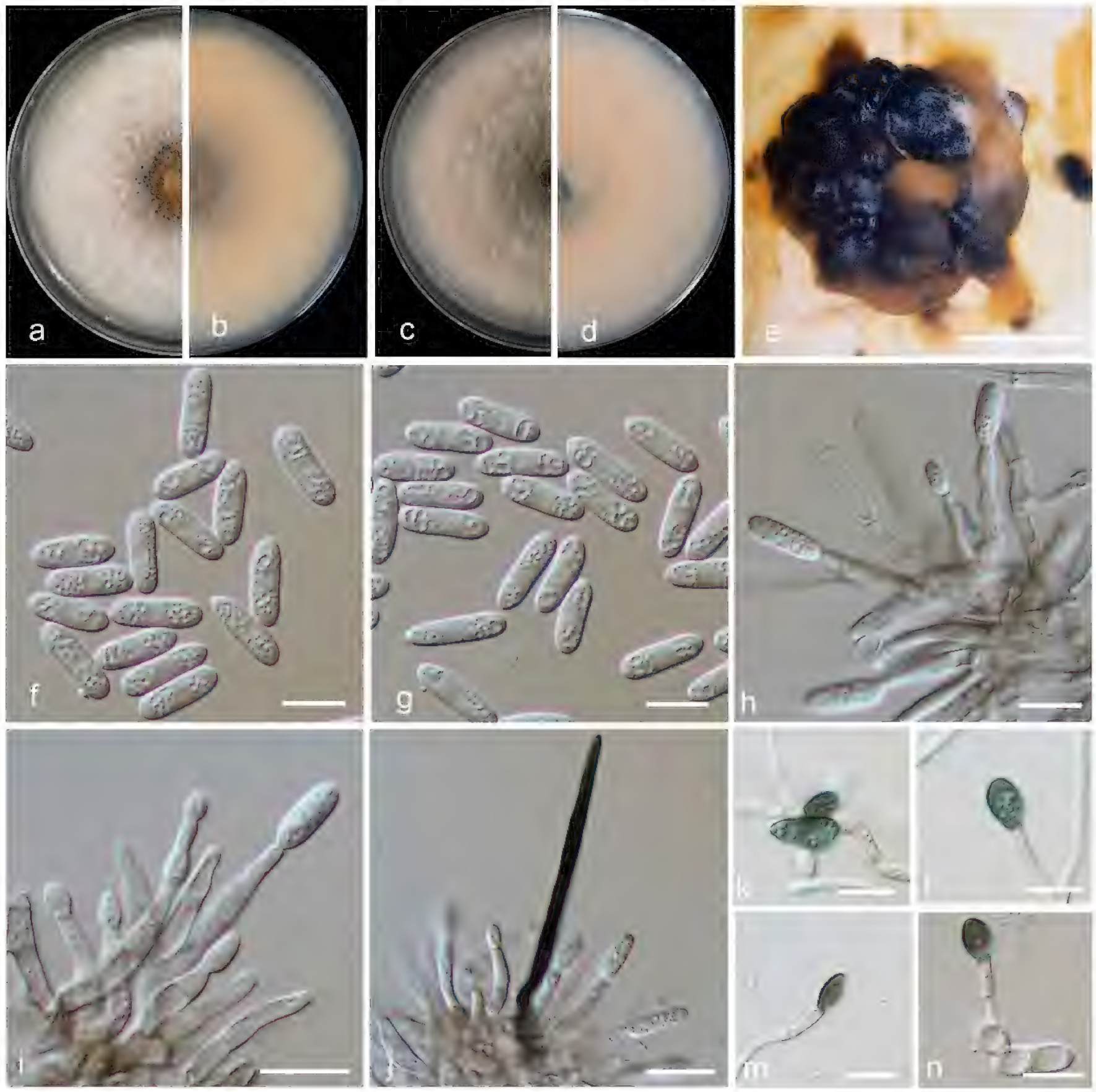

Figure 6. Morphological characteristics of *Colletotrichum chinensis* **a, b** front and back view, respectively, of 7-d-old MEA culture **c, d** front and back view, respectively, of 7-d-old PDA culture **e** conidiomata **f, g** conidia **h, i** conidiophores **j** setae **k–n** appressoria **a–n** isolate QCG-1.1. Scale bars: 10 μm (**f–n**); 500 μm (**e**).

(9/165, 5.5%), *C. peakense* (7/165, 4.2%), *C. fructicola* (6/165, 3.6%), *C. fioriniae* (4/165, 2.4%), *C. boninense* (2/165, 1.3%), *C. karsti* (2/165, 1.2%) and *C. citrulli* (2/165, 1.2%) (Suppl. material 1), of which, *C. mengyinense* was the most dominant species in Gansu Province (79%), *C. chinensis* in Beijing (47%), *C. pandanicola* in Shaanxi (41%) and *C. godetiae* (45%) in Yunnan. *Colletotrichum mengyinense* and *C. siamense* were prevalent species in Shandong Province (35%). *Colletotrichum siamense* was the only species isolated in Sichuan and Xinjiang Provinces (Fig. 2).

Pathogenicity test and virulence on walnut tissues

Koch's postulate tests on twelve *Colletotrichum* species indicated that all of them could cause walnut anthracnose. Necrotic lesions and typical orange conidial masses were observed from the inoculated site on fruits and leaves after

ten days' inoculation, whereas all control fruits remained healthy (Fig. 7). The fruit lesion length in the treatments inoculating *C. fioriniae* (mean ± SD = 19.2 ± 7.3 mm), *C. pandanicola* (mean ± SD = 17.1 ± 7.4 mm), *C. siamense* (mean ± SD = 13.8 ± 6.6 mm) and *C. juglandicola* (mean ± SD = 11.9 ± 3.0 mm) were significantly higher than those observed in all other treatments ($P < 0.05$) (Suppl. material 4). The leaf lesion length in the treatments inoculating *C. fioriniae* (mean ± SD = 23.3 ± 2.1 mm), *C. pandanicola* (mean ± SD = 22.4 ± 4.4 mm), *C. siamense* (mean ± SD = 21.8 ± 8.2 mm), *C. fructicola* (mean ± SD = 20.6 ± 1.1 mm), *C. citrulli* (mean ± SD = 20.3 ± 6.0 mm) and *C. mengyinense* (mean ± SD = 19.4 ± 3.6 mm) were significantly higher than all other treatments ($P < 0.05$) (Suppl. material 4). All these twelve inoculated *Colletotrichum* species were re-isolated from the necrotic fruits and leaves, thereby fulfilling Koch's postulates.

Discussion

In total, twelve *Colletotrichum* species within three *Colletotrichum* species complexes (namely *C. acutatum*, *C. boninense* and *C. gloeosporioides*) were identified. *Colletotrichum chinensis* was described as a species new to science in this study, while *C. boninense*, *C. citrulli* and *C. karsti* were reported from walnut for the first time. Both *C. boninense* and *C. karsti* belong to the *C. boninense* species complex. *Colletotrichum boninense* species complex comprised 29 species, some of which had a diverse host range (Talhinhas and Baroncelli 2021; Liu et al. 2022). For instance, *Colletotrichum karsti* had been reported on more than 60 plant species worldwide (Talhinhas and Baroncelli 2021). However, this was the first reported of the members of *C. boninense* species complex on walnut.

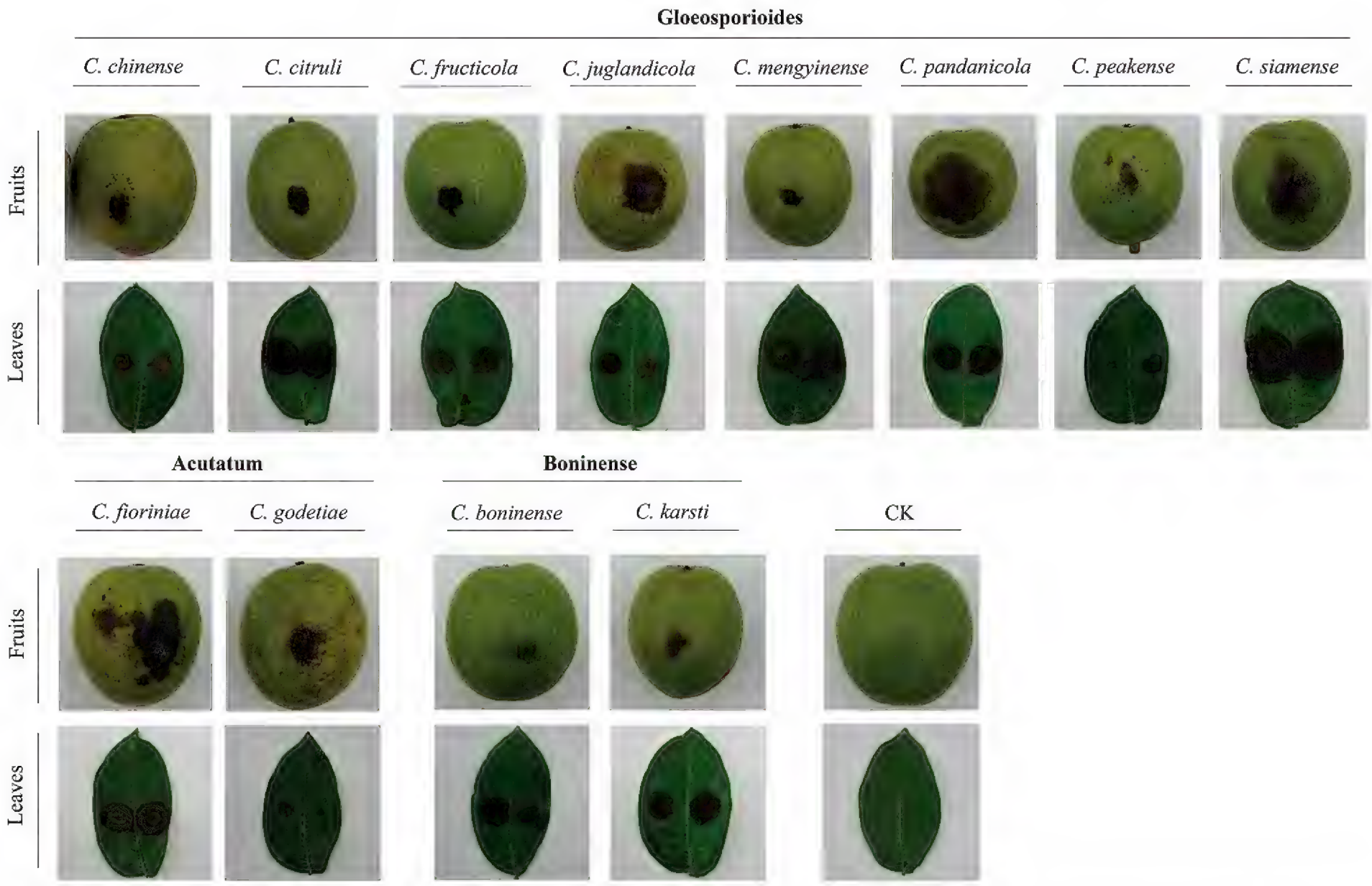

Figure 7. Symptoms of 12 *Colletotrichum* species inoculated on walnut fruits (*Juglans regia* L.) and leaves at 10 days.

All these twelve *Colletotrichum* species, isolated in this study, caused the typical symptoms of anthracnose on walnut, which led to the eventual mortality of the fruits or leaves. *Colletotrichum fioriniae*, *C. pandanicola*, *C. siamense* and *C. juglandicola* were more severe on walnut fruits than other *Colletotrichum* species, while *C. fioriniae*, *C. pandanicola*, *C. siamense*, *C. fructicola*, *C. citrulli* and *C. mengyinense* were more severe on leaves. *Colletotrichum gloeosporioides* s. s. had been reported as a more severe causal agent of walnut anthracnose than most other species in Beijing (Li et al. 2023). However, it was absent from the current study.

The geographical distribution of *Colletotrichum* spp. in China exhibited a distinct regional prevalence. For instance, *C. mengyinense* prevailed in Gansu Province, co-existing with *C. siamense* in Shandong, *C. chinensis* in Beijing, *C. pandanicola* in Shaanxi and *C. godetiae* in Yunnan. Notably, *Colletotrichum siamense* was the only species isolated in Sichuan and Xinjiang Provinces. The causal agent of walnut anthracnose appeared to vary across different sampling sites. Comparable results were documented for anthracnose diseases in *Pyrus* spp., indicating variations in species distribution and occurrence across different regions (Fu et al. 2019). The potential explanation is that the fungal susceptibility is greatly affected by the host cultivars examined, environmental conditions, weather and farming practices (Dowling et al. 2020). For instance, disease severity diminishes when temperatures exceed 35 °C, while increased precipitation can boost disease development (Iqra et al. 2022).

A variety of management methods, including cultural, biological control and chemical control, have been tried over the years to manage *Colletotrichum* spp. infecting fruit crops (Dowling et al. 2020). Despite these efforts, chemical control remains the primary method for controlling these diseases. Future research should focus on including more isolates from walnuts across different regions of China to thoroughly investigate the distribution and diversity of *Colletotrichum* species. Additionally, it should evaluate the effectiveness of biological and chemical control agents on the growth of anthracnose pathogens in the field conditions.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

YZ designed the research and revised the manuscript; LZ performed the research and wrote the manuscript; LLZ and LHY prepared the samples, conducted the molecular experiments; CL analysed the data. All authors read and approved the final manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

GenBank accession numbers of isolates included in this study

Authors: Lin Zhang, Lili Zhao, Chen Liang, Luhan Yu, Ying Zhang

Data type: docx

Explanation note: Newly-generated sequences are in bold.

Link: https://doi.org/10.3897/mycokeys.108.127734.suppl1

Supplementary material 2

The occurrence of 12 *Colletotrichum* species

Authors: Lin Zhang, Lili Zhao, Chen Liang, Luhan Yu, Ying Zhang

Data type: docx

Link: https://doi.org/10.3897/mycokeys.108.127734.suppl2

Supplementary material 3

Morphological comparisons of twelve *Colletotrichum* species in this study and their hosts in previously reported

Authors: Lin Zhang, Lili Zhao, Chen Liang, Luhan Yu, Ying Zhang

Data type: docx

Link: https://doi.org/10.3897/mycokeys.108.127734.suppl3

Supplementary material 4

Mycelial diameter of twelve *Colletotrichum* species and lesion lengths formed on walnut fruits and leaves

Authors: Lin Zhang, Lili Zhao, Chen Liang, Luhan Yu, Ying Zhang

Data type: docx

Link: https://doi.org/10.3897/mycokeys.108.127734.suppl4